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The diversity of inducible and constitutively expressed *erm(C)* genes and association to different replicon types in staphylococci plasmids

Lisbeth E. de Vries,^{1,2} Henrik Christensen¹ and Yvonne Agersø^{2,*}

¹Department of Veterinary Disease Biology; University of Copenhagen; Frederiksberg, Denmark; ²National Food Institute; Technical University of Denmark; Lyngby, Denmark

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The aim of this study was to analyze the diversity of the macrolide resistance gene, *erm(C)* in relation to structural alterations affecting the gene expression. In addition, the association of *erm(C)* to mobile genetic elements (MGEs) in staphylococci mainly from Danish pigs was investigated. In total, 78 erythromycin-resistant isolates were screened for *erm(C)* by PCR. The *erm(C)* genes incl. the upstream regulatory region were sequenced and the expression types were characterized phenotypically (agar diffusion test) and genotypically (sequence analysis). Phylogenetic analysis of *erm(C)* was compared with structural alterations affecting the gene expression. Plasmids carrying *erm(C)* from seven selected isolates were fully or partially sequenced. Thirty-seven isolates were shown to be *erm(C)* positive and *erm(C)* from pigs were all constitutively expressed, mainly caused by different sized deletions (118, 111, 107, 70, 66, 16 and 3 bp) in the regulatory region. Duplication (63 bp) and substitutions were also found to cause a constitutive phenotype. Only one horse isolate had an inducible expression type. Phylogenetic analysis showed that structural alterations have happened in different *erm(C)* allele groups and not only in one group. Furthermore *erm(C)* was found mainly on plasmids (~2.4–8 kb) and gene sequence types correlated with plasmid replication (*rep*) gene types. One *erm(C)* type was linked to an IS257 element able to circularize. In conclusion, structural alterations giving rise to constitutive expression of *erm(C)* have happened several times in the evolution of *erm(C)*. Interestingly, the diversity of *erm(C)* appears to be linked to the plasmid type or MGE carrying the gene.

Introduction

Staphylococci are part of the natural skin flora and count for some of the most important veterinary pathogens, e.g., *Staphylococcus aureus* and *Staphylococcus hyicus*.¹ A very high prevalence of macrolide-lincosamide-streptogramin B (MLS_B) resistance isolates has been found among staphylococcal isolates from animals,² and the erythromycin ribosome methylase (*erm*) gene *erm(C)* is the most predominant MLS_B resistance gene in staphylococcal isolates from both humans and animals.^{3,4} The *erm(C)* gene has mostly been found on small multi-copy plasmids (2.3–2.5 kb),⁵ but also larger and more diverse plasmids (3.7–4 kb) with mobilization (*mob*) and/or plasmid recombination (*pre*) genes have been reported.^{6–9}

The expression of *erm(C)* can be either inducible or constitutive. Macrolide antibiotics are characterized by a lactone ring containing 12–16 members, but only 14- and 15-membered macrolides like erythromycin can induce *erm(C)* expression by translational attenuation.^{10–13} This mechanism is controlled by the formation of a hairpin structure of the *erm(C)* mRNA formed by the pairing of four inverted repeat (IR) sequences located in the region upstream of *erm(C)* (Fig. 1A).^{10,12} In the absence of an

inducer, IR1 pairs with IR2 and IR3 pairs with IR4 in a two loop structure which renders the *erm(C)* start codon non-accessible to the ribosome, and only a leader peptide located just upstream of *erm(C)* is translated.¹⁰ Upon induction, inducers bind to the ribosome translating the leader, which leads to alteration in the mRNA secondary structure so that IR2 pairs with IR3, the *erm(C)* start codon becomes accessible, and *erm(C)* is translated.¹⁰ Structural alterations within the upstream region of *erm(C)* can result in constitutive expression of *erm(C)* which also confers resistance to 16-membered macrolides (e.g., tylosin) and lincosamides, streptogramin B and ketolides.^{7,9,14,15} Clinical reports and in vitro studies have shown that the gene expression can change from inducible to constitutive under selective pressure of non-inducers.^{5,15–18} Although the use of non-inducing growth promoters such as tylosin was discontinued in Denmark after 1998, tylosin is still the predominant macrolide used for therapeutic treatment of infections in pigs.^{19,20} Alterations causing constitutive expression of *erm(C)* are believed to be a result of high concentrations of non-inducible macrolides like e.g., tylosin in the environment.^{5,16}

The literature contains a number of reports of structural alterations detected in the upstream regulatory region of

*Correspondence to: Yvonne Agersø; Email: yvoa@food.dtu.dk
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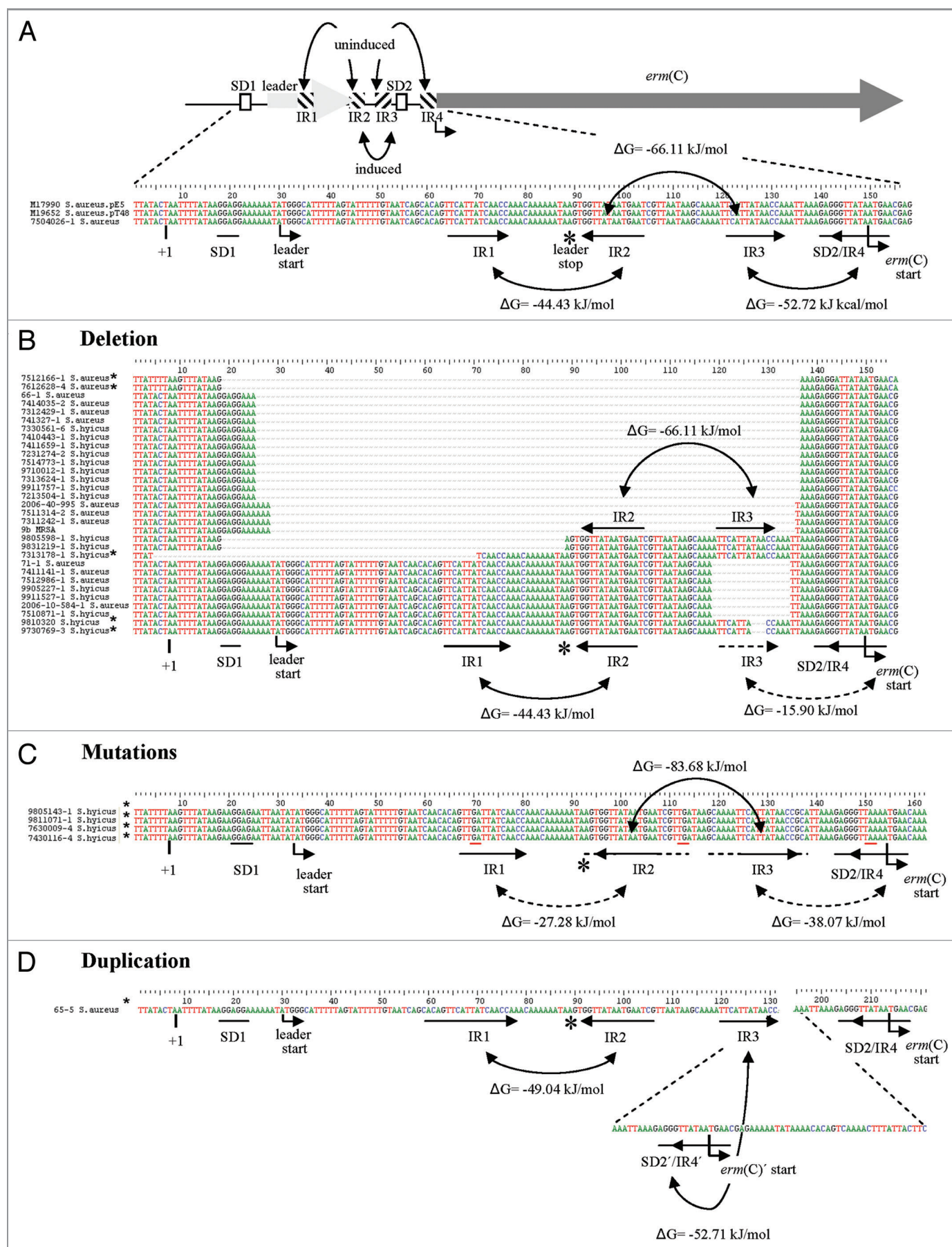


Figure 1 (See previous page). Schematic representation of the inducible *erm(C)* gene and structural alterations in the upstream regulatory region, which lead to constitutive expression of *erm(C)*. The *erm(C)* mRNA begins at +1 and besides the *erm(C)* gene (735 bp) it contains an upstream leader ORF (60 bp). Ribosomal binding sites, Shine Dalgarno sequence (SD) precedes both genes. In the absence of an inducer, the inverted repeat sequences (IR), IR1-IR4 form a double hairpin structure, rendering the *erm(C)* start codon non-accessible to the ribosome. Predicted pairing of IR sequences is indicated with arrows (threshold for predicted pairing was $\Delta G < -41.84$ kJ/mol, -10 kcal/mol) and hypothetical pairing are indicated with dotted arrows. Structural alterations that have not previously been reported are highlighted with an asterisk (*). (A) The upstream region of *erm(C)* from the *S. aureus* isolate (7504026-1) shown to have an inducible phenotype aligned with two GenBank sequences known to have an inducible phenotype. (B). The region upstream *erm(C)* containing deletions resulting in a constitutive phenotype. The alignment shows the different size deletions found in sequences from this study that had a constitutive phenotype. (C). Substitutions (underlined in read) in the upstream region of *erm(C)* from four *S. hyicus* isolates were predicted to result in the pairing of IR2 with IR3, which explains the constitutive phenotype (see text). (D) The upstream region of *erm(C)* in the *S. aureus* isolate (65-5) predicted to have a constitutive expression of *erm(C)* due to a 63 bp duplication containing a truncated *erm(C)*' including an additional SD2'/IR4'.

erm(C).^{8,14-16} Until now, three different types have been identified (Fig. 1): Sequence deletions of varying length, duplications of parts of the *erm(C)* gene as well as multiple point mutations.^{8,14-16} However, no one has studied the evolution of the *erm(C)* gene in comparison with different structural alterations causing a constitutive gene expression, and how the gene diversity is related to associated mobile genetic elements (MGEs). Thus, the aim of this study was to analyze the diversity of *erm(C)* in relation to structural alterations affecting the gene expression, and to characterize associated MGEs in staphylococci mainly from Danish pigs. Specifically, erythromycin resistant isolates were screened for the *erm(C)* gene, and a phenotypic test revealed the *erm(C)* expression types. Sequence analysis identified the genetic background for observed phenotypes. Finally, phylogenetic analysis of the *erm(C)* gene were compared with structural alterations causing a constitutive phenotype and with replicon types (*rep* gene) found on *erm(C)* carrying plasmids.

Results

Screening *S. aureus* and *S. hyicus* isolates for *erm(C)*. Out of 78 erythromycin-resistant staphylococcal isolates, 16 *S. aureus* and 21 *S. hyicus* isolates were shown to be positive for *erm(C)* (all 37 isolates are listed in Table 1). The highest prevalence of *erm(C)* was found among *S. aureus* isolates from pigs, with 60.9% compared with 40.4% among *S. hyicus* isolates.

Expression types for *erm(C)*. Agar diffusion tests showed that only one *S. aureus* horse isolate (7504026-1) had an inducible clindamycin resistance phenotype (Fig. S1), and sequence analysis showed the upstream regulatory region of *erm(C)* to be identical or highly similar to corresponding regions from isolates known to have an inducible phenotype; *S. aureus* pT48 (GenBank, M19652), pE5 (GenBank, M17990), pWG738 (GenBank, DQ088624), pE194 (GenBank, V01278), *S. hominis* pSES5 (GenBank, Y09001) and *S. lentus* pSTE2 (GenBank, AJ888003). For these sequences, IR1:IR2 ($\Delta G = -44.43$ kJ/mol, -10.62 kcal/mol) and IR3:IR4 ($\Delta G = -52.72$ kJ/mol, -12.60 kcal/mol) were predicted to pair in the absence of an inducer with a total change in Gibbs free energy of $\Delta G_{\text{total}} = -97.15$ kJ/mol (-23.22 kcal/mol) (Fig. 1A). In the presence of an inducer, IR3 and IR4 were predicted to pair, $\Delta G = -66.11$ kJ/mol (-15.80 kcal/mol) (Fig. 1A).

Thirty-six *S. hyicus* and *S. aureus* isolates (35 from pig and 1 from horse isolates) had a constitutively expressed clindamycin resistance phenotype (Table 1; Fig. S1). Sequence analysis of the upstream regulatory region showed that 31 isolates contained different size deletions (118 bp, 111 bp, 107 bp, 70 bp, 66 bp, 16 bp and 3 bp), predicted to result in alternative secondary structures of the *erm(C)* mRNA, leaving IR4 and SD2 accessible for translation of *erm(C)* (Fig. 1B). Observed deletions of size, 118 bp, 111 bp and 107 bp included IR1-IR3. Deletions of size 70 bp, 58 bp and 66 bp only included IR1, and pairing of IR2 with IR3 were predicted to be more stable ($\Delta G = -66.11$ kJ/mol, -15.80 kcal/mol) than the pairing of IR3 with IR4 ($\Delta G = -52.72$ kJ/mol, -12.60 kcal/mol) (Fig. 1A and B). Finally, deletions of size 6 bp and 3 bp included all or part of IR3. No pairing of IR3 containing a 3 bp deletion with IR4 was predicted, since the calculated change in Gibbs free energy (ΔG -15.90 kJ/mol, -3.80 kcal/mol) was much higher than the threshold for pairing (< -41.84 kJ/mol, -10 kcal/mol) (Fig. 1B).

However, four *S. hyicus* isolates (9805143-1, 9811071-1, 7630009-4, 7430116-4) contained an upstream *erm(C)* region with a complete leader sequence and four IR sequences, these were shown to have a constitutive phenotype. An agar disk diffusion test of an *E. faecalis* recipient (JH2-2) transformed with the plasmid carrying *erm(C)* from one of these four *S. hyicus* isolates (9811071-1), ruled out the possibility that another gene could have caused the observed constitutive phenotype. The sequence analysis showed that, for these four isolates, the upstream *erm(C)* region contained four IR sequences with a few substitutions (Fig. 1C) that have not been characterized previously, and these substitutions could explain the observed phenotype. The pairing of IR2:IR3 was predicted ($\Delta G = -83.68$ kJ/mol, -20.00 kcal/mol) instead of IR1:IR2 and IR3:IR4, thus leaving the IR4 and SD2 accessible for translation of *erm(C)* (Fig. 1C). Finally, one isolate that was lost (*S. aureus* 65-5) could not be tested phenotypically, but sequence analysis showed it to contain a 63 bp duplication including an additional SD2'/IR4' in the upstream regulatory region (Fig. 1D). This *erm(C)* gene was predicted to be constitutively expressed by the pairing of IR1:IR2 ($\Delta G = -49.04$ kJ/mol, -11.72 kcal/mol) and IR3:IR4' ($\Delta G = -52.71$ kJ/mol, -12.60 kcal/mol) with a total change in Gibbs free energy of $\Delta G_{\text{total}} = -101.75$ kJ/mol (-24.32 kcal/mol) and leaving IR4 and the *erm(C)* start site accessible for translation (Fig. 1C).

Table 1. Staphylococci strains carrying *erm(C)* used in this study

Strain	Species	Source /year	Other resistance phenotypes	Approx size of <i>erm(C)</i> plasmid or DNA ring ¹	<i>erm(C)</i> phylogenetic group ²	Expression type of <i>erm(C)</i> ³	GenBank accession number
9710013-1	<i>S. hyicus</i>	pig/1997	STR	2.4 kb	4a	Constitutive	JF968519
9730769-3	<i>S. hyicus</i>	pig/1997	PEN, SPT, STR, SUL, TMP	1.7 kb	2	Constitutive	JF968541
9805143-1	<i>S. hyicus</i>	pig/1998	PEN, STR, SUL, TET, TMP	3.7–4 kb	1b	Constitutive	JF968536
9805598-1 ⁴	<i>S. hyicus</i>	pig/1998	-	2.4 kb	4a	Constitutive	JF968536
9810320-1	<i>S. hyicus</i>	pig/1998	PEN, STR, TET, TMP	1.7 kb	2	Constitutive	JF968538
9811071-1	<i>S. hyicus</i>	pig/1998	PEN, SUL, TET, TMP	3.7–4 kb	1b	Constitutive	JF968540
9831219-1 ⁴	<i>S. hyicus</i>	pig/1998	-	2.4 kb	4a	Constitutive	JF968524
9905227-1 ⁴	<i>S. hyicus</i>	pig/1999	PEN, STR	2.4 kb	4a	Constitutive	JF968528
9911527-1	<i>S. hyicus</i>	pig/1999	-	2.4 kb	4b	Constitutive	JF968522
9911757-1 ⁴	<i>S. hyicus</i>	pig/1999	PEN	2.4 kb	4a	Constitutive	JF968529
7213504-1	<i>S. hyicus</i>	pig/2000	CIP, PEN, STR, TET, TMP	2.4 kb	4a	Constitutive	JF968521
7231274-2	<i>S. hyicus</i>	pig/2000	-	2.4 kb	4c	Constitutive	JF968517
7313178-1	<i>S. hyicus</i>	pig/2001	TET	2.4 kb	4a	Constitutive	JF968543
7313624-1	<i>S. hyicus</i>	pig/2001	PEN, STR, TET	2.4 kb	4a	Constitutive	JF968520
7330561-6	<i>S. hyicus</i>	pig/2001	PEN, SPT, STR, SUL, TET, TIA	2.4 kb	4a	Constitutive	JF968514
7410443-1	<i>S. hyicus</i>	pig/2002	PEN, SPT, STR	2.4 kb	4c	Constitutive	JF968515
7411659-1	<i>S. hyicus</i>	pig/2002	CIP, PEN, STR	2.4 kb	4a	Constitutive	JF968516
7430116-4	<i>S. hyicus</i>	pig/2002	PEN	3.7–4 kb	1b	Constitutive	JF968534
7510871-1	<i>S. hyicus</i>	pig/2003	PEN, STR	2.4 kb	4a	Constitutive	JF968531
7514773-1	<i>S. hyicus</i>	pig/2003	PEN, TET	2.4 kb	4a	Constitutive	JF968518
7630009-4	<i>S. hyicus</i>	pig/2004	PEN, STR	3.7–4 kb	1b	Constitutive	JF968535
7311242-1	<i>S. aureus</i> ⁵ CC398	pig/2001	SPT, STR, TET	2.4 kb	4a	Constitutive	JF968512
7312429-1	<i>S. aureus</i>	pig/2001	SPT	2.4 kb	4c	Constitutive	JF968510
7411141-1	<i>S. aureus</i> ⁵ CC9	pig/2002	PEN, TET	4 kb	3	Constitutive	JF968526
7413727-1	<i>S. aureus</i> ⁵ CC398	pig/2002	PEN, TET	2.4 kb	4a	Constitutive	JF968513
7414035-2	<i>S. aureus</i> ⁵ CC398	pig/2002	PEN, STR, TET	2.4 kb	4a	Constitutive	JF968509
7511314-2	<i>S. aureus</i> ⁵ CC398	pig/2003	SPT, TET, TMP	2.4 kb	4a	Constitutive	JF968508
7512166-1	<i>S. aureus</i> ⁵ CC398	pig/2003	PEN, STR, TET, TMP	7–8 kb	1a	Constitutive	JF968537
7512986-1	<i>S. aureus</i> ⁵ CC398	pig/2003	SPT, TET	4 kb	3	Constitutive	JF968527
7504026-1	<i>S. aureus</i> ⁵ CC30	horse/2003	PEN, STR	2.4 kb	4	Inducible	JF968532
7612628-4	<i>S. aureus</i> ⁵ CC398	pig/2004	SPT, TET	7–8 kb	1a	Constitutive	JF968539
200610584-1	<i>S. aureus</i> ⁵ CC9	pig/2006	PEN, TET, TMP	2.4 kb	4a	Constitutive	JF968530
200640995-1	<i>S. aureus</i>	horse/2006	PEN	2.4 kb	4a	Constitutive	JF968511
65-5	<i>S. aureus</i>	pig/2007	ND	ND	4a	Constitutive	JF968533
66-1	<i>S. aureus</i> ⁵ CC9	pig/2007	-	2.4 kb	4a	Constitutive	JF968507
71-1	<i>S. aureus</i> ⁵ CC398	pig/2007	PEN, TET, TMP	4 kb	3	Constitutive	JF968525
9b	<i>S. aureus</i> ⁵ CC398	pig/2007	CEF, PEN, SPT, STR, TET, TMP	2.4 kb	4a	Constitutive	JF968542

¹Determined as the approximate size of the PCR product generated with primers pointing out of the *erm(C)* gene (primers 2020 and 2021 or 2022).

²Determined by a phylogenetic analysis (Fig. 2). ³Determined by agar diffusion test, except for 65-1 that was predicted to be constitutively expressed. ⁴PFGE pattern of 9805598-1 was shown to be identical with 9831219-1 and the pattern of 9905227-1 was shown to be identical with 9911757-1 (Fig. S5). ⁵CC type that could be deduced from *spa* types (Table S1). Isolates predicted to be of CC398 represented different related *spa* types such as t034, t2876, t571. CEF, Ceftiofur; CIP, Ciprofloxacin; CHL, chloramphenicol; ERY, erythromycin; FLO, florfenicol; PEN, penicillin; SPE, spectinomycin; STR, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TIA, tiamulin; TMP, trimethoprim; ND, not determined.

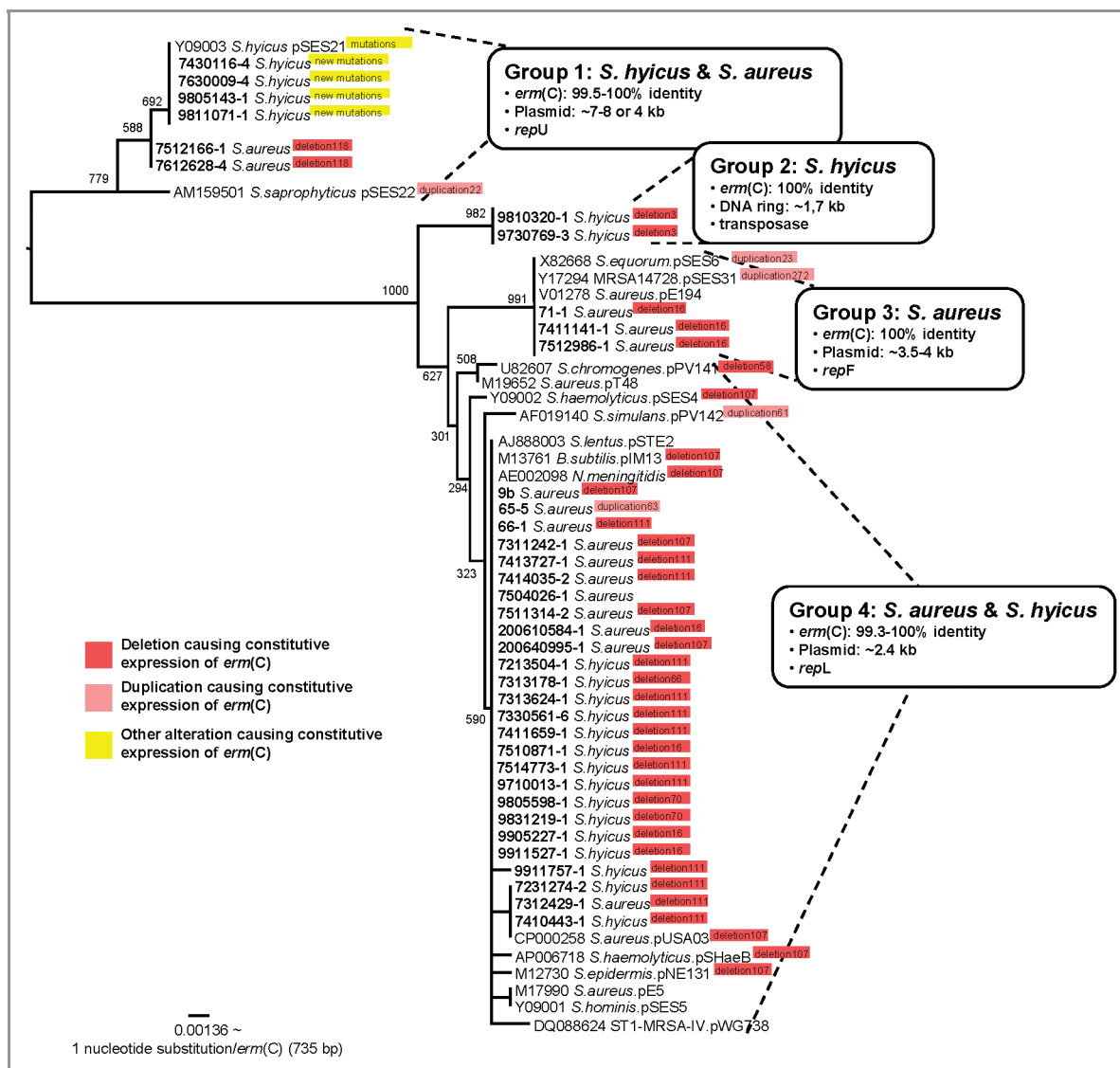


Figure 2. Phylogenetic gene tree of *erm(C)* compared with structural alterations in the upstream regulatory region of *erm(C)* conferring constitutive expression. Sequences from this study (marked in bold letters) were mainly isolated from pigs (Table 1), whereas the GenBank sequences were mainly from human isolates. Structural alterations causing constitutive expression of *erm(C)* are marked with a color code, and the type of alteration is noted with a number which indicates the size (in bp) of deletion or duplication causing the constitutive phenotype. Bootstrap values are indicated at branch points (out of 1,000 generated NJ trees). The relatively low bootstrap values at some branch points within group 4 can be explained by single nucleotide differences between sequences in this group.

Phylogenetic analysis of *erm(C)* compared with expression types. Comparing the *erm(C)* gene and the corresponding amino acid sequences from all 37 staphylococcal isolates revealed 7 unique sequence types both on DNA and amino acid level. A phylogenetic analysis of the 37 *erm(C)* gene sequences and 19 *erm(C)* GenBank sequences divided them into four *erm(C)* groups (Fig. 2). A comparison of the corresponding amino acid sequences also resulted in the same grouping of four *Erm(C)* groups (data not shown). As illustrated in Figure 2, all 56 *erm(C)* gene sequences were highly similar (95.1–100% DNA identity). The *erm(C)* sequences within groups 2, 3 and 4 were highly related (98.8–99% DNA identity) whereas *erm(C)* of group 1 were more distinct (95.1–95.9% DNA identity) compared with the other groups.

The phylogenetic tree showed structural alterations in the *erm(C)* upstream regulatory region to be located in all four *erm(C)* groups (Fig. 2). Interestingly, inducible *erm(C)* genes were only present within *erm(C)* group 3 and 4 (e.g., V01278; pE194 and M19652; pT48/ M117990; pE5) (Fig. 2). Thus complete regulatory sequences of inducible *erm(C)* genes have not been reported for sequences belonging to *erm(C)* group 1 and 2. Overall, deletions appear to be the most common form of alteration causing a constitutive *erm(C)* expression and specific type/sizes of deletions appear to be specific for the different *erm(C)* groups. Only deletions of 16 bp were present in more than one of the *erm(C)* groups; the highly related *erm(C)* group 3 and 4 (Fig. 2). Altogether, this showed that structural alterations

giving rise to constitutive expression have happened in different allele groups of *erm(C)* as opposed to in one type only.

The *erm(C)* gene sequences are linked to the plasmid carrying the gene. In order to test whether the sequenced *erm(C)* genes were located on small plasmids, which is most often the case,⁵⁻⁹ PCR reactions using primers pointing in opposite directions out of *erm(C)* (Fig. S2), were performed. If *erm(C)* was located on a plasmid or a circular DNA molecule, PCR products would be generated also when the primers were pointing in opposite directions. In this case, different size PCR products were amplified for 36 isolates (except *S. aureus* 65-5 that was lost during the study), and the product sizes were shown to correlate with the four *erm(C)* groups (Fig. 2; Table 1). Full or partial sequencing of these PCR products, representing each of the four groups, showed *erm(C)* of group 1, 3 and 4 to be located on *rep* containing plasmids of approximately 2.4–8 kb, whereas *erm(C)* of group 2 was associated with a IS257-like transposase containing element of 1.7 kb (Fig. S3).

In order to study the relationship between *erm(C)* and associated plasmids, a phylogenetic tree based on sequenced *rep* genes from this study and *rep* genes from *erm(C)* carrying plasmids available from GenBank was constructed. The *rep* genes were divided into three main groups of *repU*, *repF* and *repL* corresponding to *erm(C)* phylogenetic group 1, 3 and 4 respectively (Fig. 2; Fig. S4). Thus, the *erm(C)* gene sequences appear to be linked to the plasmid (or the mobile element) carrying them.

Discussion

The screening of erythromycin resistant staphylococci showed approximately half of the pig isolates to contain the *erm(C)* gene (60.9% of *S. aureus* and 40.4% of *S. hyicus*) which is in overall agreement with former prevalence studies.^{2,3,21}

Phenotypic testing revealed all pig isolates containing *erm(C)* to be constitutively expressed, and only one horse isolate was shown to contain an inducible *erm(C)* gene. In environments with high concentrations of non-inducing macrolides (e.g., tylosin), staphylococci with constitutively expressed *erm(C)* genes are believed to have a selective advantage both to sensitive staphylococci and to staphylococci containing a regulated *erm(C)* gene.^{5,16} Macrolides are one of the most commonly used antimicrobial agents for therapeutic treatment of infections in pigs in Denmark, and tylosin is the predominant macrolide used. Also large amounts of non-inducing lincosamides are used in pigs.²⁰ In 2008, a total of 14,181 kg macrolide and lincosamides were sold for therapeutic use in pigs in Denmark. In comparison, only about half of this use (7,600 kg of macrolide and lincosamides) was sold for therapeutic treatment in pigs in 1996 (before growth promoters were discontinued in Denmark), but the use of tylosin for growth promotion alone was 68,350 kg. Evidence suggesting that the use of non-inducing macrolides (tylosin for growth promotion) and lincosamides in domestic animals from Germany, UK and Denmark have selected for constitutively resistant *erm(C)*-carrying staphylococcal strains have also been reported.⁵ Another study has suggested that the discontinuation of tylosin use for growth promotion after 1998 in Denmark could be

reflected in the prevalence of regulated *erm(C)* genes compared with constitutively expressed genes.¹⁶ In contrast, we found that all pig isolates containing *erm(C)* (1997–2007) were constitutively expressed, and therefore our results indicate that the therapeutic usage of non-inducing macrolide and lincosamides in Denmark still play an important role in selecting for constitutively expressed *erm(C)* genes in staphylococci from pigs. Alternatively, these results may simply reflect that the main reservoir of *erm(C)* carrying strains were selected for constitutively expressed *erm(C)* genes before the ban of growth promoters, and that the therapeutic use of macrolides and lincosamides continue to select for resistance genes such as *erm(C)*. It is worth noting that the only isolate found to have an inducible *erm(C)* expression was from a horse. This may be an indication of the much lower consumption of macrolides in horses compared with pigs (in 2008, only about 1 kg of macrolides and lincosamides were sold for therapeutic use in horses compared with the 14,181 kg sold for the use in pigs).²⁰ Although, this would have to be investigated further in a study designed to compare expression types between different animal reservoirs.

Investigating the genetic basis of the observed expression types, we found mostly deletions of different size to be the cause of the constitutive phenotype. In this study, we identified structural alterations causing constitutive expression of *erm(C)* that have not previously been reported. These included deletions of 3 bp, 66 bp, 118 bp, a duplication of 63 bp, and new substitutions within the IR sequences. The latter alteration was verified to be responsible for the observed phenotype by transforming the *S. hyicus* plasmid carrying *erm(C)* (9811071-1) into an *Enterococcus* recipient strain. This strongly suggests that the *erm(C)* located on the *S. hyicus* plasmid was responsible for the observed phenotype, even if *erm(C)* induction might work slightly different in different genera.

The phylogenetic analysis illustrated that different types (sizes) of structural alterations causing a constitutive *erm(C)* expression may appear to be specific for the *erm(C)* groups, with the exception of 16 bp deletions that have occurred both in *erm(C)* groups 3 and 4. However, a larger test population would be required to draw any broader conclusions from the observed trend. A former study showed the recombination system of a host cell to play a role in the development of different types of structural alterations associated with constitutive *erm(C)* gene expression, and a model for the development of deletions in the *erm(C)* regulatory region by homologous recombination suggested the sequence region beginning just before IR3 until SD2 to be involved in a 16 bp deletion.¹⁴ The sequence in this region is identical in *erm(C)* group 2, 3 and 4 but differs from *erm(C)* group 1, which explains why 16 bp deletions have been observed in *erm(C)* group 3 and 4, but not in group 1. Replication slippage or illegitimate recombination may result in tandem duplications observed in the *erm(C)* translational attenuator.¹⁵ Altogether, our results showed that different types of alterations causing constitutive expression of *erm(C)* have happened in different *erm(C)* attenuators, and that the size and/or type of alterations that have happened appear to depend mainly on the sequence surrounding the alteration. However, it has also been suggested that the frequency and type of alteration may depend on the selecting antimicrobial agent.²²

Interestingly, this study shows that the *erm(C)* gene sequences are linked to the replicon type of the plasmid (or IS element) carrying the gene. We found *erm(C)* to be located mainly (25/36 isolates) on small *repL* containing plasmids of approx. 2.4 kb, but we also identified *erm(C)* on larger *repF* plasmids of approx. 3.8 kb (3/36 isolates) and on *repU* plasmids of approx. 4–8 kb (6/36 isolates), also containing a *prelmob* gene. In addition, *erm(C)* was also found (for 2/36 isolates) to be associated with an IS257 transposase (also known as IS431), an active staphylococcal insertion sequence^{23,24} previously found to be associated with other resistance genes in staphylococcus species,²⁵ but this is the first report of *erm(C)* associated with IS257. Regarding the gene regulations, it is worth noting that no complete regulatory sequence of an inducible *erm(C)* has been reported for *erm(C)* group 1 and 2 linked to *repU* plasmids and IS257 transposase elements, respectively. Thus it is not known whether possible structural alterations conferring constitutive expression for these two *erm(C)* types may have occurred before or after the genes were associated with their respective MGE. In fact, it remains to be proven whether these two *erm(C)* types have actually developed from an inducible gene type at all.

As illustrated by the phylogenetic tree (Fig. 2), *erm(C)* encoding plasmids have been detected in different staphylococcal species from various animal sources and also from humans. These plasmids can be spread by mobilization between members of different staphylococcal species, but also between staphylococci and bacillus.^{26–28} Most of the small *erm(C)* carrying plasmids, however, do not encode any mobilization genes. In correlation with this, the phylogenetic *erm(C)* tree illustrates that within *erm(C)* group 4, three subgroups of identical *erm(C)* sequences with identical size deletions in the upstream region have been detected both in *S. aureus* and *S. hyicus* (16 bp and 111 bp deletion) and *S. aureus*, *Bacillus subtilis* and *Neisseria meningitidis* (107 bp deletion). Thus, horizontal transfer of *erm(C)* on small plasmids without genes encoding mobilization has not only occurred between different species of staphylococci but also between bacteria from different genera. Such a transfer may have occurred by transduction or transformation,^{29,30} or alternatively mobilization proteins encoded by other plasmids may facilitate mobilization of small *erm(C)* plasmids carrying an *oriT*.^{31,32} The dissemination is believed to be controlled by the plasmids stability in the new hosts²⁸ or alternatively by the occurrence of chromosome integration, which appears to be the case for *erm(C)* observed in *Neisseria meningitidis*. In this study we also identified *erm(C)* on a mobilizable plasmid (*repU*) containing a *prelmob* gene in *S. hyicus*. This plasmid was related to previously reported plasmids from Gram positive bacteria; with a *prelmob* gene identical to pUB10, pC16, pG01, pSK41 and a recombination site, *RS_A* (*oriT*) identical to a corresponding site in *S. saprophyticus* plasmid pSES22 (AM159501). Interestingly, an almost identical region (1534 bp) from this *S. hyicus* plasmid, including *erm(C)* and starting just before the *RS_A*, (data not shown) has also been detected on a large (20 kb) plasmid from *Lactobacillus reuteri* (FJ489650) isolated from a pig in the 1970s.^{33,34} This strongly suggests that exchange of *erm(C)* between *Lactobacillus* and *Staphylococcus* has occurred. Finally,

the finding of *erm(C)* associated with an IS257 element on a circular DNA element without any *rep* gene suggests a large mobilization potential for this *erm(C)* gene, since it appears to have the ability to integrate both in chromosomal, transposon and plasmids sites containing IS257 elements.

In summary, we have shown that *erm(C)* genes in Danish staphylococci mainly from pigs are constitutively expressed, which is mainly caused by deletions in the regulatory region. Alterations giving rise to constitutive expression have happened in different allele groups of *erm(C)* and not only in one type, and the type and/or size appear to be linked to the sequence surrounding the alteration. Furthermore, *erm(C)* was found to be located mainly on small plasmids, and the gene sequence was shown to be linked to the plasmid or the element carrying the gene. Altogether this suggests the different *erm(C)* carrying plasmids found in staphylococci species until now have evolved independently.

Materials and Methods

Strains. The 78 erythromycin resistant isolates from Danish pigs, horses and lamb used in this study were identified as *S. aureus* or *S. hyicus* as described previously.³⁵ Different clones were represented among the studied isolates as shown by *spa* typing or PFGE typing (Table S1; Fig. S5). All of the 52 *S. hyicus* and 20 of the 26 *S. aureus* were different diagnostic submissions to the National Food Institute, DTU (1997–2006), which originated from farms all across Denmark. Six *S. aureus* isolates were obtained from different healthy pigs from at least two different farms (2007). As part of the standard procedure at DTU, all isolates were tested for susceptibility to ceftiofur, chloramphenicol, erythromycin, florfenicol, penicillin, spectinomycin, streptomycin, sulfamethoxazole, tetracycline and trimethoprim by using the broth microdilution Sensititer method (Trek Diagnostic Systems Ltd, UK) as described previously and following CLSI guidelines (Table 1).^{36,37} Due to a change in the standard procedure, strains isolated from 2000 and later were also tested for susceptibility to tiamulin and ciprofloxacin (Table 1). Two isolates (9b and 71-1) were positive for a *mecA* screening PCR, showing them to be methicillin-resistant *S. aureus* (MRSA) (http://www.crl-ar.eu/data/images/meca-pcr_protocol%2006.02.08.pdf)

PCR and sequencing. All the 78 erythromycin resistant staphylococcal isolates were screened for *erm(C)* by PCR as described previously² using DNA Taq polymerase (Ampliqon, Denmark) and the primers 28 and 29 (Table S2). The *Bacillus subtilis* strain B.3HU104 containing *erm(C)* on the plasmid pE194 was used as a positive control for all PCR reactions. The *erm(C)* gene including an upstream regulatory region (766–947 bp) of the 37 staphylococcal isolates that were positive for *erm(C)* (Table 1), was sequenced by Macrogen, Korea (www.macrogen.com/eng/sequencing/sequence_main.jsp) as outlined in Figure S2.

PCR reactions using the primers 2020 and 2021 or 2022 pointing in opposite directions out of *erm(C)* (Fig. S2) were performed for 36 isolates (all isolates that were positive for *erm(C)*, except for *S. aureus* 65-5). Phusion™ High-Fidelity DNA Polymerase (Finnzymes, Finland) was used with conditions

recommended by the manufacturer. Such PCR products were completely or partially sequenced for seven isolates which were selected to represent different *erm(C)* phylogenetic groups as determined by the phylogenetic analysis: For three *S. hyicus* (7313178-1, 9730769-3, 9811071-1) and two *S. aureus* (9b, 7312429-1) isolates, the PCR products were completely sequenced, and for two *S. aureus* (7512986-1, 7612628-4) the PCR products were partially sequenced. The following combinations of sequencing primers were used: For isolates 7512986-1 and 9730769-3, primers 2020, 2022 and ned234 were used, for isolates 7313178-1 and 9b, primers 2020, 2022, ned234 and gr4ned_b, for isolate 9811071-1 primers, 2020, 2021, op1SH, ned1, gr1upSH_b and gr1nedSH_SA_b and finally for isolate 7612628-4, primers 2020, 2021, gr1nedSH_SA_b and op1SA were used. All sequences were assembled, annotated and visualized in Vector NTI (Invitrogen). All primers used in this study are listed in Table S2.

Sequence analysis. GenBank was searched for full length *erm(C)* genes based on the definition that the *erm(C)* gene shares $\geq 80\%$ similarity on the amino acid level.⁴ Nineteen gene sequences were selected based on the following criteria; the upstream regulatory sequence should be sequenced and the sequences should be published in a paper containing information about the phenotypic *erm(C)* expression type. A neighbor-joining (NJ) tree based on a multiple alignment of the 37 *erm(C)* gene sequences (735 bp) obtained in this study and 19 *erm(C)* genes from GenBank was constructed in Clustal X³⁸ and visualized by MEGA 4.0.2.³⁹ The tree was rooted with the *S. aureus erm(B)* gene (GenBank, AB300568) as outgroup. Another NJ tree based on 5 replication (*rep*) genes that were identified on the full or partial sequenced *erm(C)* plasmids from this study and 14 *rep* genes located on full or partial *erm(C)* containing plasmids deposit in GenBank, was constructed as described above.

Sequence analysis of the upstream region of *erm(C)* was performed for the 37 isolates listed in Table 1 by alignment in ClustalX³⁸ and manual checking and editing of alignments in Bioedit version 7.0.0.⁴⁰ Changes in Gibbs free energy (ΔG) for predicted pairing of IR sequences in the *erm(C)* mRNA were calculated with the program RNAfold (<http://bibiserv.techfak.uni-bielefeld.de/rnafold/submission.html>) using sequence regions containing the specific IRs as queries.⁴¹⁻⁴³ Pairing of IR sequences were predicted for $\Delta G < -10$ kcal/mol (-41.84 kJ/mol). All pairwise sequence comparisons were performed with the EMBOSS program water (local alignments) and/or needle (global alignments) (<http://www.ebi.ac.uk/emboss/align/>).⁴⁴

Transforming a *erm(C)* plasmids into a recipient strain. Plasmid purification from one *S. hyicus* isolate (9811071-1) was

performed using QIAfilter Plasmid Midi Kit (Qiagen). Electrocompetent *Enterococcus faecalis*, JH2-2RF cells were transformed with purified *S. hyicus* plasmid (9811071-1) or with an *Enterococcus* erythromycin resistant plasmid, PAT18 (positive control), as described previously.⁴⁵ Transformants were selected on brain heart infusion (BHI) agar plates (Becton, Dickinson and Company, USA), supplemented with 12.5 mg/L of rifampicin and 12.5 mg/L of fusidic acid and either 4 mg/L or 8 mg/L erythromycin. Altogether, 10 transformants (five from each the BHI plates with 4 or 8 mg/L erythromycin, respectively) were selected and confirmed to carry a plasmid borne *erm(C)* by two PCRs; one *erm(C)* PCR screen using primers 28 and 29, and a long PCR using the primers 2020 and 2021 pointing out of the *erm(C)*. For one transformant (JH2-2_9811071-1 B1-RFE), the PCR product from the long PCR (primer 2020–2021) was partially sequenced with primer 2021, and the upstream region of *erm(C)* was confirmed to be identical with the corresponding region from donor *S. hyicus* (9811071-1) *erm(C)* plasmid.

Agar diffusion test of *erm(C)* expression types. For 36 staphylococcal isolates (all isolates listed in Table 1, except 65-5) and the 10 *E. faecalis* transformants (inclusive JH2-2_9811071-1 B1-RFE), expression types were tested by an agar disk diffusion test, as described previously and in accordance with the standard CLSI disk diffusion method.^{46,47} For each of the tested strains, an erythromycin disc (15 μ g) was placed in the middle surrounded by two clindamycin discs (2 μ g) (lincosamide antibiotic) within a distance of 1–2 cm on a müller-hinton plate streaked with a standard 0.5 McFarland inoculum suspension. Plates were incubated for 16 to 18 h at 37°C. Flattening of the clindamycin zone adjacent to the erythromycin disc (a “D-zone”) was considered proof for an inducible expression type.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental material may be found here: www.landesbioscience.com/journals/mge/article/20109

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